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(54) Title: METHOD OF DETECTING TUMORS CONTAINING COMPLEXES OF P53 AND HSP70

(57) Abstract

The present invention relates to the finding that the class of mutant p53 proteins which bind to HSP70 defines a group of mutant p53 proteins which elicit serum autoantibodies. Thus, disclosed is a method of classifying tumor cells for the ability to produce serum p53 autoantibodies in a patient carrying such tumor cells, a method of detecting tumor cells containing a mutant p53 protein capable of forming a complex with a 70 kilodalton heat shock protein (hsp70) in the cells in a patient, a method of distinguishing tumor cells capable of causing more aggressive disease in a patient carrying the tumor cells, and a method of monitoring a patient for the recurrence of disease in a patient previously diagnosed as carrying tumor cells.

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METHOD OF DETECTING TUMORS CONTAINING
COMPLEXES OF P53 AND HSP70

Related Applications

5   This application is a continuation-in-part of copending application Serial Number 07/869,292, filed 14 April 1992, the disclosure of which is to be incorporated herein by reference.

Field of the Invention

10   The present invention relates to the finding that the class of mutant p53 proteins which bind to HSP70 defines a group of mutant p53 proteins which elicit serum autoantibodies.

Background of the Invention

15   Mutations in the p53 tumor suppressor gene are found at a high frequency in a wide variety of primary human cancers. See, e.g., M. Hollstein et al., *Science* **253**, 49-53 (1991). Breast cancer is typical of human carcinomas with respect to p53 involvement, and it has 20 been reported that approximately 10% of breast cancer patients have circulating antibodies directed against the

p53 protein.   L. Crawford et al., *Int. J. Cancer* **30**, 403-408 (1982).   The diagnostic significance of circulating p53 antibodies has, however, been uncertain: it has subsequently been found that 21% of patients with
5 benign mammary fibroadenomas show elevated levels of the p53 protein in tumor tissue samples, and that more than half of patients with serum p53 antibodies show no elevation of the p53 protein in tumor tissue.   L. Crawford et al., *Mol. Biol. Med.* **2**, 261-272 (1984).

10   Summary of the Invention

             In the study leading to the present invention, p53 immunogenicity was examined in breast cancer patients with particular regard to the type of p53 mutant being expressed by tumor cells and the ability of these
15 proteins to bind the 70 kd heat shock protein.   As discussed in detail below, we have now found that the class of mutant p53 proteins which bind to HSP70 defines a group of mutant p53 proteins which elicit serum autoantibodies.   Because p53 proteins which bind HSP70
20 tend to be more tumorigenic, see P. Hinds et al., *Cell Growth Differ.* **1**, 571-580 (1990); O. Halevy et al., *Science* **250**, 113-116 (1990), this finding provides a way to use serum p53 auto antibodies for the diagnosis and prognosis of disease.

25   In view of the foregoing, a first aspect of the present invention is a method of classifying tumor cells for the ability to produce serum p53 autoantibodies in a patient carrying such tumor cells. The method comprises collecting a sample of tumor cells from the patient, and
30 then detecting (e.g., by coimmunoprecipitation) the presence of a complex of the p53 protein and a 70 kilodalton heat shock protein (hsp70) in the tumor cells. The presence of this complex indicates that the tumor cells are capable of eliciting serum p53 autoantibodies
35 in the patient. In one embodiment, the tumor cells are contained within a tumor (e.g., a primary tumor), and the

detecting step is carried out concurrently with the step of removing the tumor from the patient; in another embodiment, the tumor cells are contained within a tumor (e.g., a primary tumor), and the detecting step is
5   followed by the step of removing the tumor from the patient. The step of removing the tumor from the patient may optionally be followed by the step of detecting the appearance of serum p53 autoantibodies in the patient, the appearance of said autoantibodies indicating the
10 growth or presence of the tumor cells in the patient.

A second aspect of the present invention is a method of detecting tumor cells expressing a mutant p53 protein capable of forming a complex with a 70 kilodalton heat shock protein (hsp70). The method comprises
15 collecting a sample of biological fluid from the patient, and then detecting p53 autoantibodies in the biological fluid, the presence of p53 autoantibodies in the biological fluid indicating the tumor cells express a mutant p53 protein capable of forming a complex with
20 hsp70.

A third aspect of the present invention is a method of distinguishing tumor cells capable of causing a more aggressive disease in a patient carrying the tumor cells. The method comprises collecting a sample of a
25 biological fluid from the patient, and then detecting p53 autoantibodies in the biological fluid, the presence of p53 autoantibodies in the biological fluid indicating the tumor cells are capable of causing a more aggressive disease.

30   A fourth aspect of the present invention is a method of monitoring a patient for the recurrence of disease in a patient previously diagnosed as carrying tumor cells, and which patient has previously undergone treatment to reduce the number of said tumor cells. The
35 method comprises collecting a sample of biological fluid from the patient, and then detecting p53 autoantibodies in the biological fluid, the presence of p53

autoantibodies in the biological fluid indicating that
the number of the tumor cells in the patient has
increased. Typically, the treatment referred to is one
effective to reduce the amount of p53 autoantibodies in
5   the biological fluid, and the tumor cells express a
mutant p53 protein capable of forming a complex with a 70
kilodalton heat shock protein in said cells. The
detecting step may be repeated on a plurality of
occasions to provide continued monitoring for the
10   recurrence of disease. The method may optionally further
comprise the step of detecting p53 autoantibodies in the
patient prior to the treatment.

A fifth aspect of the present invention is a
prognostic method for detecting aggressive disease in a
15   patient not previously diagnosed as carrying tumor cells.
The method comprises collecting a sample of biological
fluid from the patient, and then detecting p53
autoantibodies in the biological fluid. The presence of
p53 autoantibodies in the biological fluid indicates that
20   the patient harbors tumor cells which are more
aggressive.

A sixth aspect of the present invention is a
diagnostic method for detecting cancer in a patient. The
method comprises collecting a sample of biological fluid
25   from the patient, and then detecting p53 autoantibodies
in the biological fluid. The presence of p53
autoantibodies in the biological fluid indicates that the
patient harbors tumor cells.

The foregoing and other objects and aspects of
30   the present invention are explained in detail in the
drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 shows the screening of sera from
patients with breast cancer for anti-p53 antibodies.
35   Immunoprecipitation of metabolically labeled protein
extracts from the breast cancer cell line, BT-20, with:

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PAb1801; normal human serum (nl); s15 is from a breast cancer patient whose tumor did not overexpress p53 protein; 57-ANA is from a patient with other (undefined) anti-nuclear antibodies; s11, s33, s7, s45, s39, s50, and
5 s56 are from breast cancer patients whose tumors expressed high levels of mutant p53 protein.

  Figure 2 shows the recovery of wild-type and a variety of mutant p53 proteins with sera from breast cancer patient s11. All seven antibody-positive sera
10 recognized this panel of diverse p53 proteins, in addition to wild-type murine p53 from NIH3T3 cells (data not shown).

  Figure 3 shows the co-immunoprecipitation of p53 and HSP70 only in tumors from patients with anti-p53
15 antibodies. Protein extracts from tumor tissues were immunoprecipitated with an anti-p53 antibody (P) or anti-HSP70 antibody (H) and then probed with (A) rabbit anti-HSP70 sera and (B) PAb1801.

  Figure 4 shows the results of the primary assay
20 in the enzyme immunoassay format to detect the presence of p53 autoantibodies in serum samples from normal people, and patients with colon cancer, lung cancer, and breast cancer, respectively. The 0.427 units/ml cutoff value is also shown.

25 Figure 5 shows the results of the confirmatory assay in the enzyme immunoassay format for confirming the presence of p53 autoantibodies from six serum specimens. "Ca" denotes cancer and "AUTOAB" denotes autoantibodies.

Detailed Description of the Invention

30 The method disclosed herein may be employed with patients suspected of carrying cancer (including, but not limited to, cancers of both endothelial and epithelial origin such as colo-rectal, lung, breast, or ovarian cancer tumor cells), particularly breast cancer,
35 and may be employed both to monitor patients who have been previously diagnosed as carrying cancer (including

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patients who have undergone treatment for the cancer) and to screen patients who have not been previously diagnosed as carrying cancer. Patients are typically human males and females, though veterinary applications for the

5 present invention (i.e., in the treatment of dogs, cats, horses, etc.) are also contemplated. Where previous diagnosis is carried out, the diagnosis is typically through the identification of a mass upon palpation, radiological diagnosis, cytology, or the detection of a

10 humoral marker. Treatment of the cancer is typically through cytoreductive surgery, through administration of antineoplastic agents, or combinations thereof such as cytoreductive surgery followed by treatment with antineoplastic agents.

15 As noted above, the present invention can be used for diagnosing whether a patient has cancer, and wheter it is a more aggressive form of cancer. The present invention also provides a method for subsequent monitoring of a patient for tumor recurrence. In

20 patients bearing cancers that express mutant p53 proteins, an antibody response to the p53 protein is sometimes induced. Upon removal of the tumor, the antibody response regresses. In cases where the patient forms a tumor at a secondary site, i.e., a metastasis,

25 the antibody response reappears. This antibody response is specific for patients bearing tumors that contain p53 mutations. Therefore, by assaying for the presence of the p53 antibodies, the presence or absence of the tumor can be determined. However, only a subset of p53 mutant

30 proteins induce this immune response. This subset has now been identified as those p53 mutant proteins that can form stable complexes with hsp70. The presence of this complex can be assayed in primary tumor tissue by means such as coimmunoprecipitation with antibodies to p53 and

35 hsp70. Therefore, with a sample of the primary tumor tissue, it can be predicted which patients will mount an antibody response to p53. This is useful if the primary

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tumor is too small to efficiently induce the response or the patient has been pretreated with chemotherapy or radiation which has obliterated or obscured the immune response. When the patient has recovered their immune
5   response and upon tumor recurrence, they would be expected to mount an immune response which can be readily assayed. Also, an immune response which can be assayed would occur when the primary tumor has grown to a size sufficient to cause an immune response, such is also the
10 case where secondary or metastasized tumors cause an immune response, as in the case of a relapse. If the p53/hsp70 complex is detected in the primary tumor, the patient would be monitored for the appearance of a p53 antibody response which would signal tumor recurrence.

15   The phrase "more aggressive disease" as used herein refers to (a) disease which arises from cancer cells which grow rapidly; (b) disease which arises from cancer cells which undergo rapid metastasis; and (c) disease for which there is a high probability of
20 recurrence after treatment.

Samples taken from patients for use in the detecting steps described herein are generally biological fluids such as serum, blood plasma, or ascites fluid. Serum and blood plasma, particularly serum, is preferred.
25 The patient need not be one currently harboring tumor cells, as autoantibodies may be expected to remain in a patient for some time after all cells which generated such antibodies have been removed before eventually dissipating.

30   Where samples are taken from a patient for the purpose of detecting the recurrence of disease, the samples are preferably taken at a time after the treatment (surgical, chemical, etc.) sufficient in duration for residual autoantibodies to have dissipated.
35 In general, the collecting step is carried out at least six weeks after the treatment, but may be carried out three months, six months, or even one year after the

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treatment (where the treatment involves a course of treatments, such as a course of chemotherapy, this time is measured from the last occurence thereof). Such samples may be collected from the patient periodically,
5   on several occasions, to provide continued monitoring for the recurrence of disease. The time period between taking such samples will vary depending on the type and status of disease: typically, samples are collected at intervals of from six months to one year, but when the
10   patient has undergone treatment for advanced cancer, the interval between collection may be about 3 months. Subsequent treatment regimes may, if desired, be initiated while the monitoring program initiated with the previous treatment is still being carried out.

15   The step of detecting p53 autoantibodies in the sample of biological fluid may be carried out in accordance with known techniques. *See, e.g.,* L. Crawford et al., *Int. J. Cancer* 30, 403-408 (1982); L. Crawford et al., *Mol. Biol. Med.* 2, 261-272 (1984). Examples of
20   suitable assays include radioimmunoassay, immunofluorescence assays, enzyme-linked immunoassays, and the like. Those skilled in the art will be familiar with numerous specific immunoassay formats and variations thereof which may be useful for carrying out the method
25   disclosed herein. *See generally* E. Maggio, *Enzyme-Immunoassay*, (1980)(CRC Press, Inc., Boca Raton, FL).

      The present invention is explained in greater detail in the following examples. The examples show,
30   among other things, that (1) mutant p53 proteins which bind HSP70 elicit serum autoantibodies; (2) conversely, the presence of anti-p53 autoantibodies reflects the presence of a p53 mutation which binds hsp70; (3) the presence of an anti-p53 autoantibody therefore
35   establishes the presence of a growth within the body of a subject which produces an hsp70-binding mutant p53; (4) detection of an anti-p53 autoantibody can be used to

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determine the class of p53 mutant produced within the body; (5) detection of an anti-p53 autoantibody will determine that a growth somewhere in the body is cancerous and is producing an hsp70-binding p53 mutant
5 protein; and (6) detection of p53 autoantibody indicates a more aggressive tumor. In example 1, Jack Keene, Eric Winer and Robert Bast of Duke University, Durham, North Carolina, supplied patient sera. Temperatures are given in degrees Centigrade unless otherwise indicated.

10   EXAMPLE 1

I. MATERIAL AND METHODS

    <u>Tissue.</u> Pieces from breast biopsies and mastectomies performed at Duke University Medical Center were collected after surgical removal, immediately flash
15 frozen, and stored at -120°C. Specimens from sixty patients whose tumors were diagnosed as primary invasive breast carcinoma by a member of the Department of Pathology were studied. Thirty of these tumors had been shown, by immunohistochemical analysis, to overexpress
20 the p53 protein, while thirty expressed normal, low levels of the protein.

    <u>Cell lines.</u> The established cell lines HBL100, BT-20, BT-474, T47D and MDA-MB-468 were obtained from the American Type Culture Collection. HBL100 is an SV40-
25 transformed human breast epithelial cell line which expresses wild-type p53 (24); BT20, BT474, T47D and MDA-MB-468 are human breast cancer-derived cell lines that express p53 proteins with mutations at codons 132, 285, 194, and 273 respectively. See J. Nigro et al., *Nature*
30 **342**, 705-708 (1989); J. Bartek et al., *Oncogene* **5**, 893-899 (1990). The I3 cell line, obtained from Dr. Cathy A. Finlay (Department of Molecular Biology, Princeton University), is a rat embryo fibroblast line immortalized by the murine mutant p53 clone LTRcG-val[135].
35 P. Hinds et al., *J. Virol.* **63**, 739-746 (1989). Cells were grown at 37°C in RPMI 1640 supplemented with bovine

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insulin (10 µm/ml), glutamine (300mg/L), and 10% fetal
calf serum. Normal human mammary epithelial cells (HMEC)
were collected following reduction mammoplasties and
maintained in short term culture in accordance with
5 standard techniques. See V. Band & R. Sager, *Proc. Natl.*
Acad. Sci. USA **86**, 1249-1253 (1989).

Antibodies. Serum from patients with primary,
invasive breast cancer was obtained at the time of
diagnosis and stored at -20°C. PAb1801 (Ab-2, Oncogene
10 Science) is a murine anti-p53 monoclonal antibody which
reacts specifically with human p53 at an epitope between
amino acids 32 and 79. L. Banks et al., *Eur. J. Biochem.*
159, 529-534 (1986). PAb421 is a murine anti-p53
monoclonal antibody which reacts with an epitope of
15 mammalian p53 between amino acids 370 and 378, A. Wade-
Evans & J. Jenkins, *EMBO J.* **4**, 699-706 (1985), and is
produced by a hybridoma cell line obtained from Dr.
Arnold J. Levine (Department of Molecular Biology,
Princeton University). HSP72/73 (Ab-1, Oncogene Science)
20 is a murine monoclonal antibody which reacts with HSP70
proteins in mammalian cells (clone W27, E. Harlow).
Rabbit antiserum directed against human C-terminal
epitopes representing the last 21 amino acids of at least
the hsc70 and hsp70 members of the heat shock protein
25 family, Philip Hinds et al., *Mol. Cell Biol.* **7**, 2863-2869
(1987), was obtained from Dr. P. Hinds (Department of
Molecular Biology, Whitehead Institute). TA-1, an anti-
HER2/neu murine IgG$_1$ monoclonal antibody (DuPont), normal
human serum, and serum from fifteen patients with anti-
30 nuclear antibodies associated with autoimmune disorders
(obtained from Dr. J. D. Keene, Department of
Microbiology, Duke University) were used as control
antibodies.

Sequence analysis. Sequencing of the highly
35 conserved region of the p53 gene from mRNA was performed
in accordance with known techniques. *See, e.g.,* A.
Davidoff et al., *Proc. Natl. Acad. Sci. USA* **88**, 5006-5010

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(1991). Briefly, 1 μg of total RNA was used as a template for p53 first strand cDNA synthesis by MuLV reverse transcriptase (Bethesda Research Lab) using an anti-sense oligonucleotide from exon 10 as a primer.

5 Exons 4 through 10 were then amplified using the polymerase chain reaction by adding an oligo primer from exon 4 and Taq DNA polymerase (Promega). The 712 bp product of this reaction was gel purified, reamplified, and purified again by filtration through a Sepharose CL-

10 6B (Pharmacia) spin column, ethanol precipitated, and resolubilized in water. This material was the template for dideoxy sequencing using Sequenase 2.0 (United States Biochemical). Oligonucleotides flanking each of the exons 5, 6, 7 and 8 were used to prime the reactions

15 which were performed by first boiling the primer-template mix, labeling on ice for 10 min with ^{32}P-dATP and then running the termination reactions at 45°C for 10 min. The products were electrophoresed on a polyacrylamide gel which was then soaked in 10% acetic acid/12% methanol,

20 dried and set with Kodak XAR film.

Immunoprecipitation. Cell lines were metabolically labelled with 50 μCi/ml ^{35}S-methionine (Amersham) for 2 h. At the end of the labelling period, cells were scraped, homogenized in lysis buffer (50 mM

25 Tris, 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF) and then sonicated for 10 sec at 5 W/S. The supernatants were collected following centrifugation at 100,000g and preadsorbed with protein G-Sepharose (Pharmacia) for 1 hour at 4°C. Supernatants were collected following

30 centrifugation at 12,000g and incorporation was quantitated by trichloroacetic acid (TCA) precipitation. Immunoprecipitation of p53 was performed from 5 x 10^6 TCA precipitable counts from each cell line. The lysates were incubated at 4°C for 1 h with 1 μl of human serum or

35 0.1 μg of a monoclonal antibody and the immune complexes were then bound with 20 μl protein G-Sepharose (4°C, 2 h). The Sepharose was washed three times with lysis

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buffer and the samples were denatured by boiling for 5
minutes in an equal volume of SDS-loading buffer (100 mM
Tris pH = 6.8, 4% SDS, 0.2% bromophenol blue, 20%
glycerol, 50 mM β-ME) and loaded onto a 10% SDS-
5   polyacrylamide gel. Following electrophoresis of the
samples, the protein was transferred to a nitrocellulose
membrane (Schleicher and Schuell) by electroblotting at
400mA for 2 h. The membrane was dried and set with Kodak
XAR film overnight.

10   **Immunoblotting.** Protein extraction from
unlabeled cell pellets and frozen tissues was performed
as described above except that tissues were homogenized
using a Polytron (Brinkman). 250 μg of unlabeled total
protein (2 mg for HMEC and NIH3T3 cells) was reacted with
15   patient sera or monoclonal antibodies, the recovered
protein electrophoresed on an SDS-polyacrylamide gel and
then transferred to a nitrocellulose membrane also as
described above. The membrane was then treated in a
blocking solution (3% bovine serum albumin (BSA), 0.2%
20   Tween 20, 0.02% sodium azide and 1mM sodium iodide in
phosphate-buffered saline [PBS]) overnight and probed for
90 min at 37°C with PAb 421 supernatant diluted 1:3 in 5%
BSA. After washing with PBS, the blot was probed with
biotinylated $F(ab')_2$ goat anti-mouse sera (Tago) in 5 ml
25   5% BSA for 1 h. Following a final wash in PBS, antibody
binding was visualized with an avidin conjugated
immunoperoxidase detection system (Vector).

Coimmunoprecipitation. Coimmunoprecipitation
of p53 and HSP70 in tumors from patients with primary
30   invasive breast cancer was performed by first reacting
protein extracts (250 μg) from tumor tissues with either
an anti-p53 antibody or anti-HSP70 antibody. Four
immunoprecipitation reactions, two with each antibody,
were performed on protein extracts from each tissue. For
35   detecting HSP70 in the complex, immunoprecipitation was
performed with 0.1 μg of the murine monoclonal
antibodies, PAb1801 and HSP72/73. For detecting

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complexed p53, immunoprecipitation was performed with one of the human anti-p53 antisera, s11, and the rabbit anti-HSP70 antiserum. These immunoprecipitates were electrophoresed and blotted as described above. Probing

5 for HSP70 was performed with 10 μl rabbit anti-HSP70 sera in 5% BSA. Probing for p53 was performed with 1.0 μg PAb1801. Heterologous species antisera were used for these immunoprecipitations since the mouse monoclonals themselves are detected as ≈50 kd proteins with the

10 biotinylated goat anti-mouse IgG detection system. Binding was detected with biotinylated goat anti-rabbit (Vector) or anti-mouse as appropriate; visualization was again performed with an immunoperoxidase detection system.

15 **II. RESULTS**

Detection of anti-p53 antibodies. Sera collected in the perioperative period from 30 patients whose breast cancer expressed high levels of p53 and 30 patients whose tumor expressed undetectable p53 protein

20 were screened for the presence of anti-p53 antibodies. 1 μl of each serum was reacted with metabolically labeled protein lysate from the established human breast cancer cell line BT20. This cell line expresses high levels of a p53 protein harboring a mutation at codon 132. See J.

25 Bartek et al., Oncogene 5, 893-899 (1990). Sera from seven patients with tumors that overexpressed p53 contained antibodies that immunoprecipitated a 53 kd protein which co-migrated with p53 immunoprecipitated with the monoclonal antibody, PAb1801 (Figure 1).

30 Dilutional analysis of these sera showed >10-fold variation in their reactivity, with the strongest ones comparable to the immunoglobulin affinity-purified pAB1801. No band corresponding to a protein of this molecular weight was immunoprecipitated by any of the

35 sera from patients with p53-negative tumors. In addition no 53 kd protein was recovered from the BT20 lysate with

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sera from 15 patients with autoimmune disorders who were known to have anti-nuclear antibodies.

Immunoblotting was performed in order to confirm that each of these seven sera recognized the p53 oncogene. Unlabeled protein extracts were immunoprecipitated with each sera, electrophoresed, transferred to a solid support, and then probed with PAb421. The specific recovery of a 53 kd protein detected by the p53 monoclonal antibody in each case confirmed the identity of the immunoprecipitated protein (Figure 2). Immunoprecipitation from different established human and murine cell lines revealed that each of the antisera recognized a wide range of p53 proteins, both mutant and wild-type (Fig. 2).

p53 mutations correlate with immune response. Several possibilities could explain why only a subset of breast cancers expressing mutant p53 proteins elicit p53 antibodies. The amount of mutant protein may be highly variable and, after cell death, attain immunogenicity only in those tumors with high levels. However, by immunohistochemistry no major differences in the levels of p53 expression were observed in these tumors (data not shown). Alternatively, only certain mutant p53 proteins may be immunogenic even though the antibodies themselves are not mutant specific. To test this, direct sequencing of PCR amplified p53 cDNA was performed from 15 breast cancers overexpressing the protein. In each case, a mutation was found in a highly conserved region of the gene which altered the coding sequence of the protein (Table 1). A generally consistent clustering of these mutations was observed: Each patients who had not mounted an antibody response to p53 had a tumor that contained a mutation in exons 7 or 8 of the p53 gene. Conversely, five of seven patients who were antibody-positive had tumors with mutations in exons 5 or 6. The two exceptions were patients 33 and 45. Their tumors contained mutations at codons 275 (exon 8) and 238 (exon

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7) yet the patients mounted strong immune responses. In both cases, the mutations changed a cysteine residue that might be predicted to alter the long-range tertiary structure of the protein.

5

TABLE 1

Characterization of Mutant p53 Proteins with Respect to
The Site of Gene Mutation, Immunogcnicity, and the
Ability to Complex with HSP70 in 15 Breast Cancers

Patient	Gene Mutation		Anti-p53 Antibodies	HSP Binding
	Sequence (Amino Acid)	(Codon exon)		
7	TAT(Y) --> TGT(C)	205 (6)	+	+
11	TAC(Y) --> TGC(C)	163 (5)	+	+
33	TGT(C) --> GCT(R)	275 (8)	+	+
39	CGC(R) --> CAC(H)	175 (5)	+	+
45	TGT(C) --> CGT(R)	238 (7)	+	+
50	Deletion	174-181 (5)	+	+
56	CGC(R) --> CAC(H)	175 (5)	+	+
3	CCT(P) --> GCT(A)	278 (8)	-	-
12	CGT(R) --> TGT(C)	273 (8)	-	-
13	CGG(R) --> CAG(Q)	248 (7)	-	-
17	CGG(R) --> CTG(L)	282 (8)	-	-
24	ATC(I) --> AAC(N)	254 (7)	-	-
43	ATG(M) --> ATA(I)	237 (7)	-	N.D.
55	TAC(Y) --> TGC(C)	234 (7)	-	-
63	GGC(G) --> GAC(D)	245 (7)	-	-

25   **Mutant proteins that induce an antibody response complex with HSP70.** A similar clustering is evident in the human and murine mutant p53 proteins that have been analyzed for heat shock binding in tissue culture cells. p53 proteins containing mutations in exon

30 5 bind to a 70 kd heat shock protein while several

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proteins with mutations located in exon 8 fail to complex. *See* P. Hinds et al., *Cell Growth Differ.* **1**, 571-580 (1990); O. Halevy et al., *Science* **250**, 113-116 (1990). Given the possible function of an HSP70 in antigen presentation, it was of interest to determine whether the immunogenic p53 mutants formed complexes with this heat shock protein *in vivo.* Protein extracts from 14 of the 15 tumors containing p53 mutations were immunoprecipitated with anti-p53 and anti-heat shock antibodies. After blotting, separate membranes were probed with anti-p53 and heat shock antibodies in order to detect co-precipitation of these proteins. Tumor tissue from all seven patients with circulating p53 antibodies contained co-precipitating p53 and 70 kd heat shock proteins (**Figure 3**). Conversely, no p53/heat shock protein complexes were detected in tissues from the seven patients lacking detectable p53 antibodies even though levels of both p53 and HSP70 comparable to the antibody-positive tissues were present. So, it does not appear to be an absence of either p53 or HSP70 that explains the lack of the complex. Since there are several species of 70 kd heat shock protein (i.e., hsp, hsc, and hsx) that are not distinguished using these antisera, it is possible that tissues which lack the complex do not synthesize the appropriate member of the heat shock protein family. However, different mutants have been shown, in rodent fibroblasts, to have different intrinsic abilities to complex with hsc70, see P. Hinds et al., *supra;* O. Halevy et al., *supra,* suggesting that a property intrinsic to p53 is responsible for complex formation in these breast cancers.

The findings in this study suggest a method for discriminating two classes of p53 mutations in human tumors. Several lines of evidence support the idea that this may be a functionally important way to categorize mutations. p53 mutations that bind heat shock protein are more potent dominant transforming genes in

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conjunction with *ras.* P. Hinds et al., *Cell Growth Differ.* 1, 571-580 (1990). It is possible that the heat shock protein/p53 complex facilitates p53-mediated transformation by sequestration of the wild-type tumor suppressor protein, perhaps more avidly than mutant p53 proteins that fail to complex with heat shock. There also appears to be a difference in the ability of mutant p53 proteins to function in transcriptional activation. A GAL4/p53 fusion protein containing a mutation at codon 273 (human) retains this function while a mutation at codon 135 (murine) destroys the ability to transactivate a GAL4 target sequence. L. Raycroft et al., *Science* **249**, 1049-1051 (1990); S. Fields & S. Jang, *Science* **249**, 1046-1049 (1990).

The difference between HSP-binding and - nonbinding p53 mutants in their ability to transform fibroblasts may, however, support the belief that inherited p53 mutations found in patients with Li-Fraumeni syndrome result in less potent oncogenes. These mutations appear to cluster around codon 250 of the p53 gene (S. Srivastava et al., *Nature* **348**, 747 (1990); D. Malkin et al., *Science* **250**, 1233 (1990)), and would therefore be predicted not to bind HSP and, perhaps, to be less oncogenic. Patient 63 from this study contained a mutation identical to one found in a Li-Fraumeni family (codon 245, Gly→Asp) and did not bind HSP in the breast cancer. This might explain why many of these patients can reach young adulthood before manifesting the characteristic malignancies of the syndrome despite having germ-line p53 mutations.

EXAMPLE 2

The following presents an immunoassay method for detecting the presence of p53 autoantibody in a patient's biological fluid, such as serum. This immunoassay method will be called the primary assay.

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Also presented is a modified version of the primary assay which functions as a confirmatory test and will be called the confirmatory assay. A sample which tested positive for p53 autoantibodies by the primary assay can be

5 retested by the confirmatory assay to determine whether the sample is a true positive or a false positive. The general methods for conducting these assays, such as sandwich assays and competitive assays, are known to one skilled in the art. In the specific instance of this

10 invention, the primary assay has two essential components: (1) solid phase bound p53 antigens (herein designated p53S), and (2) labelled antibodies directed to human immunoglobulins. The confirmatory assay includes the two components in the primary assay plus a third

15 component: free (i.e. non-solid phase bound) competing p53 antigens (herein designated p53C).

Potential candidates for both p53S and p53C are wildtype or mutant full length native or recombinant p53 proteins and fragments thereof. Preferably, the

20 fragments are natural, recombinant, or chemically synthesized polypeptides or peptides that are predominantly recognized by p53 autoantibodies. For example, these p53 proteins or fragments can be obtained from crude cell extracts or purified from naturally

25 occurring or recombinant cells or cell lines. Examples of the sources for p53 proteins are cancer derived cell lines expressing different forms of mutant p53 proteins. For example, in the confirmatory assay described below under the section: "II. CONFIRMATORY ASSAY USING THE EIA

30 FORMAT", inhibition of p53 autoantibody binding was observed with an extract of SW620 cells which provided p53C. Another source of p53C is the T-47D cell line (designated ATCC HTB 133, available from the American Type Culture Collection) which was derived from a breast

35 tumor and expresses a mutant p53 protein in which a phenylalanine residue has been substituted for a leucine residue at codon 194. p53C has the additional

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requirement that it must not contain the crossreactive epitopes further described below with regard to p53S.

The solid phase which binds the p53S can be any of those used for immunoassays. They include natural and synthetic polymers, gels, and particles. The polymers can be cellulose or cellulose derivatives, fiberglass, and vinyl chloride. Labelled anti-human immunoglobulin antibodies are commercially available or can be made by one skilled in the art. The antibodies can be labelled with enzymatic, radioactive, fluorescent, or chemical labels.

In the primary assay, the patient's biological fluid is incubated over the solid phase, to which the p53S have been bound. The incubation period should be sufficiently long to allow the binding of the solid phase bound p53S to any anti-p53 autoantibody (herein designated ∂p53) that may be present in the patient sample to form a solid phase complex of {(p53S) (∂p53)}. Next, the unbound reagents are separated, e.g. the unbound reagents are dissolved in an aqueous medium and washed away from the solid phase, leaving behind the complex of {(p53S) (∂p53)} bound to the solid phase. Next, the solid phase is incubated with labelled anti-human immunoglobulin antibodies (herein designated *∂IgG) for a sufficient time to form the complex {(p53S) (∂p53) (*∂IgG)} which is bound to the solid phase, for patient samples containing p53 autoantibodies. This is followed by another separation step to remove the unbound reagents, leaving behind the solid phase bound {(p53S) (∂p53) (*∂IgG)} complex. The formation of the complex is detected by the label on the anti-human immunoglobulin antibodies. Thus, the formation of the {(p53S) (∂p53) (*∂IgG)} complex is directly proportional to the amount of p53 autoantibodies in the patient's serum.

It is to be noted that instead of having two incubation and wash steps (two-step assay), a single incubation and wash step (one-step assay) is also

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possible by simultaneously incubating the solid phase bound p53S, the *∂IgG and the patient's sample for a period sufficient for the formation of the solid phase bound {(p53S) (∂p53) (*∂IgG)} complex before washing off

5 the unbound reagents and detecting the presence of the complex. Further, in both the one-step and two-step assays, one can also determine the amount of the remaining unbound *∂IgG, which would be inversely proportional to the presence of the p53 autoantibodies in

10 a patient's sample.

If a patient's sample tests positive in the primary assay, it may be retested in the confirmatory assay. If the sample contains autoantibodies specific to p53 antigen, it will be identified as a true positive by

15 the confirmatory assay. Likewise, if the sample contains antibodies which are not specific to p53 antigens but crossreact with p53S, it will be identified as a false positive by the confirmatory assay. The immunoglobulins in a patient's sample could crossreact with p53S for two

20 reasons: 1) by its nature, the p53S used may contain crossreactive epitopes, e.g., a recombinant p53 protein may contain artificially introduced extraneous non-p53 structures (which facilitate the regulation, expression, or recovery of the p53 protein in cell culture

25 productions) to which the patient's immunoglobulins bind; or 2) the binding of p53S to the solid phase may alter the structure of p53S such as to create crossreactive epitopes which are not natural to a p53 antigen.

The confirmatory assay is performed similarly

30 to the primary assay except that the patient's sample is also incubated with the free competing p53 antigens (i.e. p53C). The patient's sample would be tested in two duplicate tests which are similar in all respects except that the first test contains p53C whereas the second test

35 does not (the latter serves as the negative control). Thus, the second test can be the same as the primary assay except that in the confirmatory assay, this second

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test is run side-by-side with the first test. Alternatively, the second test can contain a control protein, cell extract, polypeptide, or peptide which does not cross-react with p53 autoantibodies. This protein, cell extract, polypeptide, or peptide will not be p53 protein or fragments thereof nor resemble p53 protein or fragments thereof such as would cross-react with p53 autoantibodies. Preferably, the form of the control, be it protein, cell extract, polypeptide, or peptide, is the same as the form used for p53C in the first test. That is, if the first test uses a p53 peptide fragment or a full length p53 as p53C, the second test will use a peptide fragment or a protein of equal length which is unrelated to p53. Similarly, if the first test uses a cell extract which contains p53 proteins as a source of p53C, the second test would use a cell extract which does not contain p53 proteins, as shown in the example below. In the first test, the patient's sample is premixed with p53C and then incubated with p53S. The p53C must not contain the crossreactive epitopes described above with regard to p53S. A source of p53C may therefore be a human cell line which produces high levels of native (be they wildtype or mutant) p53 antigens. One such cell line is SW620. During the incubation with p53S, the p53C would compete with the solid phase bound p53S for binding to the autoantibody specific for p53 antigens. The p53C would not compete with the solid phase bound p53S for binding to immunoglobulin crossreactive with p53S. It will be noted that the p53C should be used in an amount which would optimally inhibit the formation of the ((p53S) (∂p53) (*∂IgG)) complex. In the case of the confirmatory assay described herein, this amount could be determined, for example, by running the confirmatory first and second tests using patient samples which are known to contain p53 specific autoantibodies (the latter can be determined, for example, by immunoprecipitation described

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in Example 1) and comparing the results from the first and second tests. A 50 to 100% inhibition is preferred.

In the second test, the patient's sample is incubated with p53S in the absence of p53C. This test

5 serves as the negative control. If the patient's sample contains autoantibody specific for the p53 antigen, the first test (which contains p53C) will have reduced formation of {(p53S) (∂p53) (*∂IgG)} complex as compared to its negative control counterpart and the sample will

10 be identified as a true positive. If the patient's sample contains immunoglobulin crossreactive with p53S, the first test will not have reduced formation of the complex as compared to its negative control counterpart and the sample will be identified as a false positive.

15 Further, the confirmatory assay can be run alone, without the primary assay, to identify a patient's sample as a "true positive" for p53 autoantibody.

The above discussion and the following detailed description also show that if the solid phase bound p53S

20 does not have epitopes which would cross-react with immunoglobulins that are not specific for p53 antigens, then the primary assay can be successfully used with this p53S without having to run a confirmatory assay.

Having described the primary and confirmatory

25 assays in general, the following are specific examples of the assays as used in an enzyme immunoassay (EIA) format.

I. PRIMARY ASSAY FOR p53 AUTOANTIBODY USING THE EIA FORMAT

(1)   Immulon-4 microtiter plates (Dynatech

30 Laboratories, Inc., Chantilly, VA) were coated with 100 μl of coating solution per well at 37C for 2 hours. The coating solution contained 0.2 μg/ml recombinant human wild type p53 protein in 0.05 M sodium bicarbonate/carbonate, pH 9.4. The recombinant p53 binds

35 monoclonal antibodies PAb1801, PAb240, and PAb421 (Catalog Numbers Ab-2, Ab-3, and Ab-1, respectively, from

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Oncogene Sciences, Inc., Uniondale, N.Y). After the coating incubation, the wells were washed three times with wash buffer (0.05% Tween-20, 0.01% SDS, and 0.01% Thimerosal in phosphate buffered saline (PBS)). Then the

5   wells were overcoated with 200 μl per well of 1% bovine serum albumin (BSA) in 0.01 M sodium bicarbonate/carbonate, pH 9.4 for 1 hour at 37° C. After the overcoating step, the wells were washed as described above.

10   (2)   To the wells were then added 100 μl of serum specimens which had been diluted 1000 fold in specimen diluent. These serum specimens were from patients with no clinical symptoms, patients with benign conditions, and patients with various cancers. The

15   specimen diluent was at pH 6.8 and contained PBS and Tris as buffers; ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid (EGTA) and ethylenediaminetetraacetic acid (EDTA) as metal chelators; Tween 20 as detergent to minimize nonspecific

20   binding of human immunoglobulins; sodium azide as preservative; fetal calf serum and normal goat serum as protein stabilizers and to minimize nonspecific binding. Each diluted specimen was tested in replicate. Also, replicate wells were loaded with 100 μl of specimen

25   diluent only which would yield "background" signal, 100 μl of a low positive control, and 100 μl of a high positive control. The positive controls were dedicated serum specimens positive for p53 autoantibody which had been diluted in specimen diluent to yield a "low"

30   positive value and a "high" positive value, respectively, in the EIA. After all samples had been loaded in wells, the plate was covered and incubated overnight (16 to 18 hrs.) at room temperature.

          (3)   After the overnight incubation, the wells

35   were washed four times with wash buffer. The wells were then loaded with 100 μl of conjugate solution. The conjugate solution was 0.2 μg/ml of goat anti-human IgG

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(H+L)-horseradish peroxidase conjugate (Catalog Number 04-10-06, Kirkegaard & Perry) in a conjugate diluent of pH 7.2. The conjugate diluent contained PBS and HEPES as buffers; benzyl alcohol as preservative; Triton x 100 as

5 a detergent; fetal calf serum and normal goat serum as protein stabilizers and to minimize nonspecific binding.

The plates were incubated 2 hrs. at 37° C. After the conjugate incubation, the wells were washed four times as described above.

10   (4) After washing, the wells were loaded with 100 μl of OPD (ophenylenediamine-2HCl) substrate solution. The OPD substrate solution was prepared by dissolving one OPD tablet (In Vitro Test No. 7181E, Abbott Laboratories, Abbott Park, IL) per 5 mls. of

15 diluent for OPD (In Vitro Test No. 5695, Abbott Laboratories, Abbott Park, IL). Plates were incubated in the dark at room temperature for 30 mins. after which 100 μl of 1 N sulfuric acid were added per well. The absorbances of the wells were read at 492 nm using the

20 Microplate Autoreader (#EL310, Bio-Tek Instruments, Burlington, VT).

      (5) Averages of replicate absorbance values for each sample were calculated. The average "background" signal was subtracted from each sample

25 average signal to correct for background absorbance. The corrected sample absorbances were divided by the corrected absorbance of the low positive control. These results yielded the p53 autoantibody values in units/ml of the serum specimens tested at a 1000-fold dilution.

30 Serum specimens which, at a 1000-fold dilution, yielded an off-scale reading were further diluted to give on-scale readings, and the readings were readjusted to reflect the units/ml values for a 1000-fold dilution.

      (6) A cutoff value of 0.427 units/ml was

35 established for the primary assay which is the mean plus four standard deviations of 73 normal serum specimens. Serum specimens yielding values equal to or greater than

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0.427 units/ml were scored positive for p53 autoantibody
and the result of the assays is shown in **Table 2** as
follows:

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Table 2

SPECIMENS	TOTAL TESTED	# POSITIVE FOR P53 AUTOAB	% POSITIVE FOR P53 AUTOAB
NORMALS	73	0	0
BENIGN CONDITIONS			
Lung Benigns	26	3	11.5
Pneumonia	20	0	0
Lipemic	12	1	8.3
Rheumatoid Factor	38	2	5.3
**GI Benign	26	2	7.7
Ulcerative colitis	20	0	0
Cirrhosis	12	0	0
Diverticulitis	10	0	0
Pancreatitis	10	0	0
MALIGNANCIES			
Colon Cancer, CEA Negative	84	13	15.5
Colon Cancer, CEA Positive	57	17	29.8
Gastric Cancer	29	4	13.8
Lung Adenocarcinoma	59	11	18.6
Small Cell Lung Cancer	52	12	23.1
Breast Cancer	66	7	10.6
Ovarian Cancer	43	8	18.6
Head & Neck Cancer	44	7	15.9
Prostate Cancer	46	7	15.2

*Autoab denotes autoantibodies
**GI denotes gastrointestinal.
Positive specimens ranged from 0.433 to 761 units/ml.

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The cutoff values in future assays may be different, depending on the number of normal sera tested and the population from which such normal sera are drawn. Preferably, increased number of normal sera are used, and

5 the sera are obtained from normal individuals who are representative of the patient population tested. Figure 4 shows an example of p53 autoantibody values for a population of 34 normal individuals, 50 colon cancer, 43 lung cancer, and 32 breast cancer patients. In this

10 specimen population, positive p53 autoantibody values ranged from just above the 0.427 units/ml cutoff value to 8.2 units/ml.

II. CONFIRMATORY ASSAY USING THE EIA FORMAT

(1) Serum specimens which tested positive for p53

15 autoantibody in the primary assay described above were subjected to testing in the confirmatory assay. The confirmation assay procedure was essentially the same as that of the above EIA primary assay except for the preparation of the serum specimen (in step 2 of the above

20 primary assay.). The serum specimen was diluted 1000 fold as described but added to it was a cancer cell line extract at a final concentration of 2 mg/ml extract protein. Each serum specimen, including the low and high positive controls, was tested this way in the presence of

25 each of two different cell extracts. One cell extract was prepared from SW620 cells (Leibovitz, et al., *Cancer Res.* 36:4562 - 4569 (1976); the cell line has been deposited and designated ATCC CCL 227, available from American Type Culture Collection, Rockville, MD). This

30 cell line was derived from lymph node metastasis of a primary colon cancer and expresses a mutant p53 protein in which a histidine residue has been substituted for an arginine residue at codon 273. The other extract was prepared from Saos-2 cells (designated ATCC HTB85,

35 available from American Type Culture Collection, Rockville, MD). This cell line was derived from

osteosarcoma and it does not express any p53 protein because both p53 genes have been deleted.

(2) The above cell extracts were prepared as follows: Freshly grown cells were washed three times in
5 cold PBS. Washed cells were suspended in cold lysis buffer at 50 X 10^6 cells/ml. (Lysis buffer is 50 mM Tris-HCl, pH 7.2, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40 (NP-40, commercially available from Sigma Chemical Company, St. Louis, MO), 1 mM phenylmethylsulfonyl
10 fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin, 5 mM NaF, and 1 mM Na_3O_4V.) The cellular suspension was sonicated for 15 sec. and then centrifuged at 100,000 X g for 1 hr. Supernatants were carefully removed and used as the cell extracts. Protein of the extracts were
15 determined via the Bradford protein method (Catalog Number 500-0006, Bio-Rad Laboratories, Richmond, CA).

(3) The diluted serum specimen in the presence of cell extract was loaded in the wells and the EIA was taken to completion as described above for the primary
20 assay (i.e. from steps 2 through 4 of the primary assay.). The principle of the confirmation EIA is that if the serum specimen contains autoantibody specific to p53, the SW620 extract will significantly decrease the EIA value compared to the Saos-2 extract. It is the
25 mutant p53 protein in the SW620 extract which will specifically inhibit binding of the p53 autoantibody to the recombinant p53 protein coated on the wells. The Saos-2 extract will have no effect because it contains no p53 protein to compete for autoantibody binding to the
30 recombinant p53 protein coated on the wells. If the serum specimen contains immunoglobulins which crossreact with the recombinant p53 protein coated on the wells, the SW620 extract will not significantly decrease the EIA value compared to the Saos-2 extract.

35 (4) After the confirmation EIA was run, the p53 autoantibody values in units/ml for each serum specimen tested in the presence of the SW620 and Saos-2

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extracts were calculated as described above in step 5 of the primary assay. One modification was that all of the corrected sample absorbances were divided by the corrected absorbance of the low positive control tested in the presence of Saos-2 extract to yield units/ml p53 autoantibody. For each serum specimen the units/ml p53 autoantibody value in the presence of SW620 extract was subtracted from the units/ml p53 autoantibody value in the presence of Saos-2 extract. This difference was divided by the serum specimen's units/ml p53 autoantibody value in the presence of Saos-2 extract and multiplied by 100 to yield % inhibition. A serum specimen is scored a true positive for p53 autoantibody if 50 to 100% inhibition was observed. Table 3 summarizes the results of confirmatory assays of specimens which initially tested positive:

Table 3

SPECIMENS	TOTAL TESTED IN CONFIRMATORTY	# CONFIRMED TRUE POSITIVE	# CONFIRMED FALSE POSITIVE
BENIGN CONDITIONS			
Lung Benigns	2	0	2
Lipemic	1	0	1
Rheumatoid Factor	1	0	1
GI Bleed	2	0	2
MALIGNANCIES			
Colon Cancer	27	25	2
Gastric Cancer	4	4	0
Lung Adenocarcinoma	13	12	1
Small Cell Lung Cancer	8	8	0
Breast Cancer	7	6	1
Ovarian Cancer	7	7	0

Some serum specimens when tested in the confirmatory assay showed 30 to 50% inhibition. This range of inhibition will require further study to determine if it is indicative of specific p53 autoantibody.

5   Figure 5 shows the results of the confirmatory EIA for several individual specimens which, except for the normal specimen, had tested positive in the primary assay for p53 autoantibody. The two benign specimens shown, a lung benign and a GI benign, gave p53 autoantibody values of 1.32 and 1.136 units/ml respectively in the 10 presence of Saos-2 extract. These values were not reduced in the presence of SW620 extract thus identifying them as false positives. In contrast the values of the three cancer specimens in the presence· of SW620 extract were significantly reduced as indicated by the percentage reduction which prove that they were true positives.

15   The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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THAT WHICH IS CLAIMED IS:

1. A method of classifying tumor cells for the ability to produce serum p53 autoantibodies in a patient carrying such tumor cells; comprising:

5   collecting a sample of tumor cells from said patient; and then

detecting the presence of a complex in said tumor cells; said complex comprised of the p53 protein and a 70 kilodalton heat shock protein (hsp70);

10   the presence of said complex indicating said tumor cells are capable of eliciting serum p53 autoantibodies in said patient.

2. A method according to claim 1, wherein said detecting step is carried out by coimmunoprecipitation.

3. A method according to claim 1, wherein said detecting

15 step is carried out concurrently with or after the step of removing said tumor from said subject.

4. A method according to claim 1, wherein said detecting step is carried out to determine the presence of tumor cells before treating the patient for said tumor.

20   5. A method according to claim 1, wherein said detecting step is followed by the step of monitoring said patient for the appearance of serum p53 autoantibodies, the appearance of said autoantibodies indicating the growth of said tumor cells in said subject.

25   6. A method according to claim 1, wherein said tumor cells are of epithelial origin.

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7. A method according to claim 1, wherein said tumor cells are selected from the group consisting of colo-rectal, lung, breast, and ovarian cancer tumor cells.

8. A method of diagnosing a tumor in a patient by
5   detecting tumor cells expressing a mutant p53 protein capable of forming a complex with a 70 kilodalton heat shock protein (hsp70) in said cells in a patient, the method comprising:
       collecting a sample of biological fluid from said patient; and then
10   detecting p53 autoantibodies in said biological fluid, the presence of p53 autoantibodies in said biological fluid indicating said tumor cells contain a mutant p53 protein capable of forming a complex with hsp70.

9. A method according to claim 8, wherein said
15   biological fluid is selected from the group consisting of serum, blood plasma, and ascites fluid.

10. A method according to claim 8, wherein said biological fluid is selected from the group consisting of serum and blood plasma.

20   11. A method according to claim 8, wherein said tumor cells are selected from the group consisting of colo-rectal, lung, breast, and ovarian cancer tumor cells.

12. A method according to claim 8, wherein said tumor cells are breast cancer tumor cells.

25   13. A method according to claim 8, wherein said patient has previously been diagnosed as carrying said tumor cells.

14. A method according to claim 8, wherein said patient has previously been diagnosed as carrying said tumor cells and undergone treatment to reduce the number of said tumor cells.

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15. A method according to claim 8, wherein said detecting step is carried out by an assay selected from the group consisting of radioimmunoassay, immunofluorescence assay, and enzyme immunoassay.

5   16. A method of distinguishing tumor cells capable of causing more aggressive disease in a patient carrying tumor cells, the method comprising:

collecting a sample of biological fluid from said patient; and then

10   detecting p53 autoantibodies in said biological fluid, the presence of p53 autoantibodies in said biological fluid indicating said tumor cells have greater tumorigenic potential and are capable of causing more aggressive disease.

17. A method according to claim 16, wherein said
15 biological fluid is selected from the group consisting of serum, blood plasma, and ascites fluid.

18. A method according to claim 16, wherein said biological fluid is selected from the group consisting of serum and blood plasma.

20   19. A method according to claim 16, wherein said tumor cells are selected from the group consisting of colo-rectal, lung, breast, and ovarian cancer tumor cells.

20. A method according to claim 16, wherein said patient has previously been diagnosed as carrying said tumor cells.

25   21. A method according to claim 16, wherein said patient has previously been diagnosed as carrying said tumor cells and undergone treatment to reduce the number of said tumor cells.

22. A method according to claim 16, wherein said detecting step is carried out by an assay selected from the group
30 consisting of radioimmunoassay, immunofluorescence assay, and enzyme

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immunoassay.

23. A method of detecting the recurrence of disease in
a patient previously diagnosed as carrying tumor cells, which patient
has previously undergone treatment to reduce the number of said tumor
5 cells, comprising:

collecting a sample of biological fluid from said
patient; and then

detecting p53 autoantibodies in said biological fluid,
the presence of p53 autoantibodies in said biological fluid
10 indicating that the number of said tumor cells in said patient has
increased.

24. A method according to claim 23, wherein said
treatment is effective to reduce the amount of p53 autoantibodies in
said biological fluid.

15 25. A method according to claim 23, wherein said
collecting step is repeated on a plurality of occasions to provide
continued monitoring for the recurrence of disease.

26. A method according to claim 23, wherein said
collecting step is carried out at least six weeks after said
20 treatment.

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27. A method according to claim 23, wherein said collecting step is carried out at least six months after said treatment.

28. A method according to claim 23, further comprising the step of detecting p53 autoantibodies in said patient prior to said treatment.

29. A method according to claim 23, wherein said tumor cells contain a mutant p53 protein capable of forming a complex with a 70 kilodalton heat shock protein in said cells.

30. A method according to claim 23, wherein said biological fluid is selected from the group consisting of serum, blood plasma, and ascites fluid.

31. A method according to claim 23, wherein said biological fluid is selected from the group consisting of serum and blood plasma.

32. A method according to claim 23, wherein said tumor cells are selected from the group consisting of colo-rectal, lung, breast, and ovarian cancer tumor cells.

33. A method according to claim 23, wherein said detecting step is carried out by an assay selected from the group consisting of radioimmunoassay, immunofluorescence assay, and enzyme immunoassay.

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34. A prognostic method for detecting aggressive disease in a patient not previously diagnosed as carrying tumor cells, comprising:

collecting a biological sample from said patient; and

5 then

detecting p53 autoantibodies in said biological sample, the presence of p53 autoantibodies in said biological sample indicating that said patient harbors a more aggressive form of tumor.

35. A method according to claim 34, wherein said

10 biological sample is selected from the group consisting of serum, blood plasma, ascites fluid and tissue.

36. A method according to claim 34, wherein said biological sample is selected from the group consisting of serum and blood plasma.

15 37. A method according to claim 34, wherein said tumor cells are selected from the group consisting of colo-rectal, lung, breast, and ovarian cancer tumor cells.

38. A method according to claim 34, wherein said detecting step is carried out by an assay selected from the group

20 consisting of radioimmunoassay, immunofluorescence assay, and enzyme immunoassay.

39. A diagnostic method for detecting cancer in a patient, comprising:

collecting a biological sample from said patient; and

25 then

detecting p53 autoantibodies in said biological sample, the presence of p53 autoantibodies in said biological sample indicating that said patient harbors tumor cells.

40. A method according to claim 39, wherein said

30 biological sample is selected from the group consisting of serum, blood plasma, ascites fluid, and tissue.

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41.   A method according to claim 39, wherein said biological sample is selected from the group consisting of serum and blood plasma.

42.   A method according to claim 39, wherein said tumor
5 cells are selected from the group consisting of colo-rectal, lung, breast, and ovarian cancer tumor cells.

43.   A method according to claim 39, wherein said detecting step is carried out by an assay selected from the group consisting of radioimmunoassay, immunofluorescence assay, and enzyme
10 immunoassay.

44. A method according to claim 39, wherein the presence of said p53 autoantibodies indicates that the patient has a more aggressive form of tumor than a patient diagnosed to have tumor caused by p53 mutation but which mutant p53 does not elicit p53
15 autoantibody response.

45.   A method of evaluating the aggressiveness of a cancer by assaying for p53 autoantibody in a patient, the presence of the p53 autoantibody indicating a more aggressive cancer.

46.   An immunoassay for quantifying p53 autoantibody (∂p53) in a human biological fluid sample, comprising the steps of:

      (a)   conducting a first test in which the sample is exposed to a solid phase bound p53 antigen (p53S), an anti-human immunoglobulin antibody (∂IgG), and a free p53 antigen (p53C) for a sufficient time to allow for the formation of a complex comprising {(p53S) (∂p53) (∂IgG)}, wherein the p53C does not cross-react with human immunoglobulin not specific to a p53 protein;

      (b)   measuring the amount of the {(p53S) (∂p53) (∂IgG)} complex;

      (c)   conducting a second test in which the sample is exposed to the solid phase bound p53 antigen (p53S) and the anti-human immunoglobulin (∂IgG), in the absence of the free p53 antigen (p53C), for a sufficient time to allow for the formation of a complex comprising {(p53S) (∂p53) (∂IgG)}; and

      (d)   measuring the amount of the {(p53S) (∂p53) (∂IgG)} complex in step (c) and comparing this amount with that of step (b).

47.   An immunoassay for detecting or quantifying p53 autoantibody (∂p53) in a human biological fluid sample, comprising the steps of:

      (a)   exposing the sample to a solid phase bound p53 antigen (p53S), and an anti-human immunoglobulin antibody (∂IgG) for a sufficient time to allow for the formation of a complex comprising {(p53S) (∂p53) (∂IgG)} and measuring the amount of this complex;

      (b)   if the amount of the {(p53S) (∂p53) (∂IgG)} complex is above a certain cut-off level, exposing the sample to the solid phase bound p53 antigen (p53S), the anti-human immunoglobulin antibody (∂IgG), and a free p53 (p53C) for a sufficient time to allow for the formation of a complex comprising {(p53S) (∂p53) (∂IgG)}, wherein the p53C does not cross-react with human immunoglobulin not specific to p53 protein.

48.   The immunoassay of claim 47, further comprising the step of:

       measuring the amount of the {(p53S) (∂p53) (∂IgG)} complex in step (b), and comparing this amount with that of step (a).

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49. The immunoassay of claim 47 further comprising the steps of:

(c) conducting a second test simultaneously with the test in step (b), in which the sample in the second test is exposed to the solid phase bound p53 antigen (p53S), the anti-human immunoglobulin (∂IgG), in the absence of the free p53 antigen (p53C), for a sufficient time to allow for the formation of a complex comprising {(p53S) (∂p53) (∂IgG)}; and

(d) measuring the amounts of the {(p53S) (∂p53) (∂IgG)} complexes in steps (b) and (c) and comparing these amounts.

50. The immunoassay of claim 46, wherein the p53S and p53C are selected from the group consisting of: wildtype or mutant full length native or recombinant p53 proteins and native, recombinant, or chemically synthesized polypeptide or peptide fragments of p53 proteins that are predominantly recognized by p53 autoantibody.

51. The immunoassay of claim 50, wherein the p53C is obtained from a naturally occurring cell or cell line which expresses a wildtype or mutant p53 protein.

52. The immunoassay of claim 50, wherein the p53C is produced by cell line SW620 or T-47D.

53. The immunoassay of claim 52, wherein the p53S is a recombinant wildtype p53.

54. The immunoassay of claim 46, wherein:

(a) the ∂IgG is labelled for detection;

(b) the p53S and p53C are either incubated with the sample simultaneously or sequentially, either p53S or p53C can be incubated before the other;

(c) the meaurement of the {(p53S) (∂p53) (∂IgG)} complex is conducted after a separation step in which the non-complexed ∂p53, p53C, and ∂IgG have been separated from the {(p53S) (∂p53) (∂IgG)} complex.

-40-

55. The immunoassay of claim 46, wherein the biological fluid is selected from the group consisting of: serum, blood plasma, and ascite fluid.

56. The immunoassay of claim 49, wherein:

5   (a)   the ∂IgG is labelled for detection;

(b)   the p53S and p53C are either incubated with the sample simultaneously or sequentially, either p53S or p53C can be incubated before the other;

(c)   the meaurement of the {(p53S) (∂p53) (∂IgG)} complex

10 is conducted after a separation step in which the non-complexed ∂p53, p53C, and ∂IgG have been separated from the {(p53S) (∂p53) (∂IgG)}. complex.

57.   An immunoassay for the detection of cancer, comprising the steps of:

15   (a) obtaining a biological fluid sample from a patient suspected of having cancer;

(b)   exposing the sample to a solid phase bound p53 antigen (p53S), and an anti-human immunoglobulin antibody (∂IgG) for a sufficient time to allow for the formation of a complex comprising

20 {(p53S) (∂p53) (∂IgG)}, wherein the p53S does not cross-react with human immunoglobulin not specific to p53; and

(c)   measuring the amount of the {(p53S) (∂p53) (∂IgG)} complex.

58. The immunoassay of claim 57, wherein:

25   (a)   the ∂IgG is labelled for detection; and

(b)   the meaurement of the {(p53S) (∂p53) (∂IgG)} complex is conducted after a separation step in which the non-complexed ∂p53 and ∂IgG have been separated from the {(p53S) (∂p53) (∂IgG)} complex.
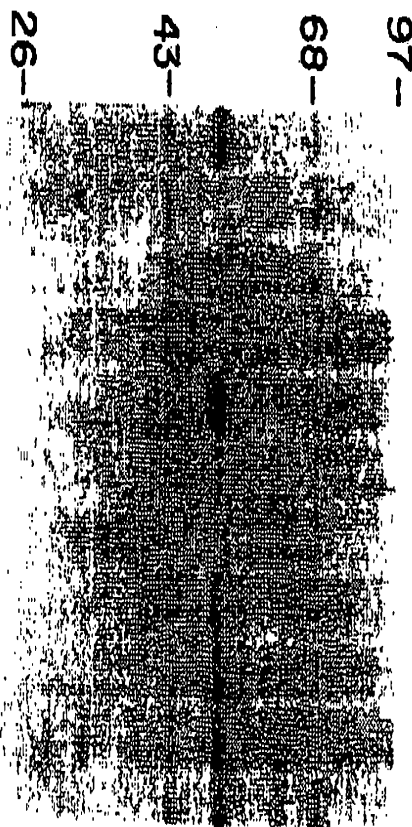
59. The immunoassay of claim 58, wherein the biological

30 fluid is selected from the group consisting of: serum, blood plasma, and ascite fluid.

FIG. 1.

97—
68—
43—
26—

1801
Nl.
S15
57–ANA
S11
S33
S7
S45
S39
S50
S56

FIG. 2.

97—
68—
43—
26—

HBL–100/1801
HBL–100/NL.
HBL–100
BT–474
T47D
MDA–MB–468
BT–20
HMEC
13

S11

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FIG. 3.

FIG. 4.

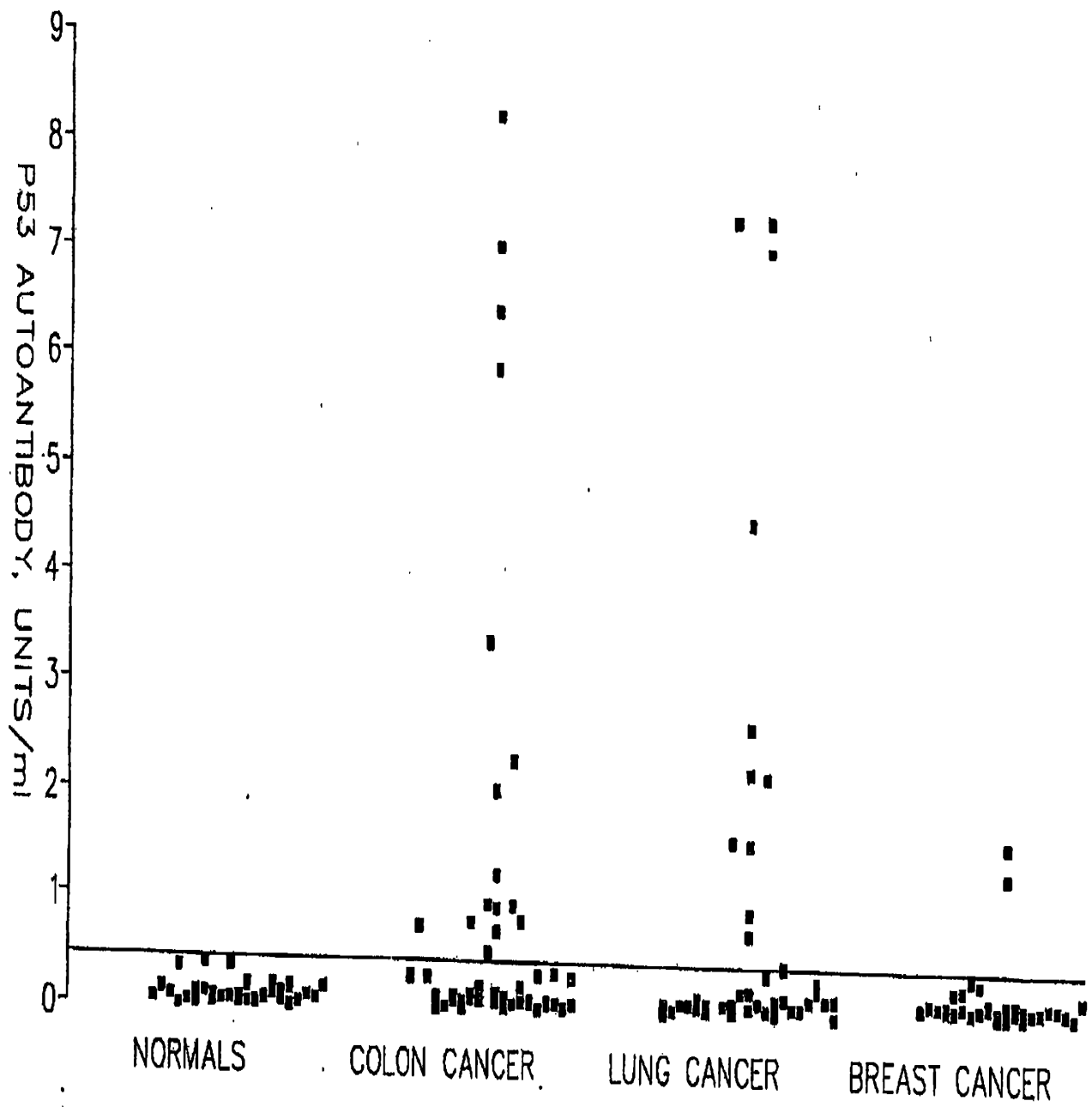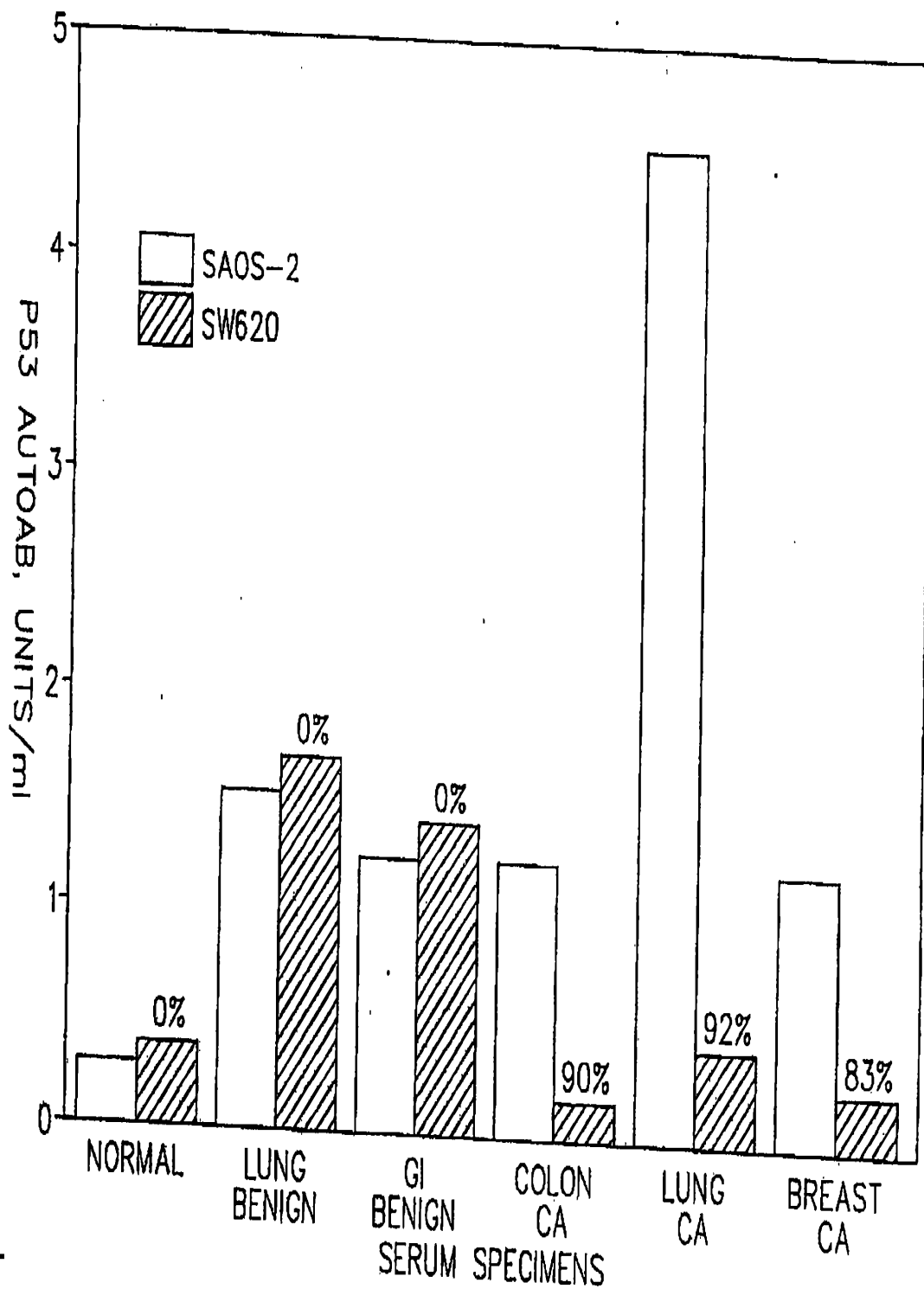FIG. 5.

International application No.
99913314
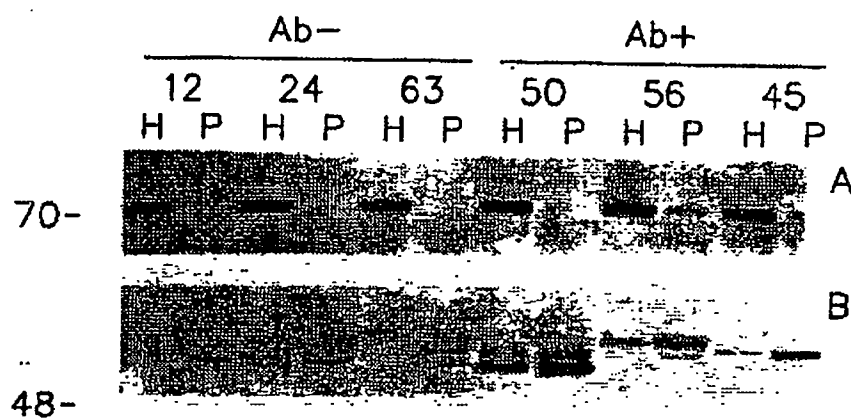
PCT/US93/02831

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(5) :G01N 33/574, 33/564, 33/545, 33/50

· US.CL :435/7.23, 7.8; 436/64, 503, 506, 531

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

  U.S. : 435/7.23, 7.8, 7.93, 7.95; 436/64, 503, 506, 531, 539

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

  APS, DIALOG

  search terms: p53, antibod?, anti-p53, autoantibod?, heat shock protein?, hs?-70

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL JOURNAL OF CANCER, Vol. 39, issued 1987, Caron de Fromentel et al, "Presence of Circulating Antibodies Against Cellular Protein p53 in a Notable Proportion of Children with B-cell Lymphoma", pages 185-189, see entire document.	8-22; 34-45
Y		5, 23-33
X	MOL BIOL MED, Vol. 2, issued 1984, Crawford et al, "The Cellular Protein p53 in Human Tumours", pages 261-272, see entire document.	8-45, 57-59
Y		5, 46-56

☒	Further documents are listed in the continuation of Box C.	☐	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 MAY 1993	23 JUN 1993

Name and mailing address of the ISA/US	Authorized officer
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INTERNATIONAL SEARCH ODOCP

International application No.

PCT/US93/02831

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P Y,P	CANCER RESEARCH, Vol. 52, issued 01 August 1992, Winter et al, "Development of Antibodies Against p53 in Lung Cancer Patients Appears to be Dependent on the Type of p53 Mutation", pages 4168-4174, see entire document.	8-11, 13-22, 39-43 5, 23-33, 46-56
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, Vol. 89, issued April 1992, Davidoff et al, "Immune Response to p53 is Dependent Upon p53/HSP70 Complexes in Breast Cancers", pages 34-39-3442, see entire document.	1-13, 15, 39-43
Y	CANCER RESEARCH, Vol. 51, issued 01 August 1991, Lehman et al, "p53 Mutations, ras Mutations, and p53-Heat Shock Protein Complexes in Human Lung Carcinoma Cell Lines", pages 4090-4096, see entire document, especially page 4091, Table 1, especially p53 mutation at codon 237.	1-7
Y	CELL GROWTH AND DIFFERENTIATION, Vol. 1, issued December 1990, Hinds et al, "Mutant p53 DNA Clones from Human Colon Carcinomas Cooperate with ras in Transforming Primary Rat Cells: A Comparison of the 'Hot Spot' Mutant Phenotypes", pages 571-580, see entire document, especially page 572, Col 1.	1-7, 46-56
Y	US, A, 4,937,185 (Webb et al) 26 June 1990, see especially paragraph bridging Col 3-4.	23-33
Y	J.A. Bellanti, "IMMNUOLOGY", published 1971, W.B. Saunders Company (Philadelphia), pages 49-54, see pages 49-54.	23-33
Y	INTERNATIONAL JOURNAL OF CANCER, Vol. 30, issued 1982, Crawford et al, "Detection of Antibodies Against the Cellular Protein p53 in Sera from Patients with Breast Cancer", pages 403-408, see entire document, especially page 404, Figure 1.	46-56
Y	P. Tijssen, "PRACTICE AND THEORY OF ENZYME IMMUNOASSAYS", published 1985, Elsevier (Amsterdam), pages 481-483, see pages 481-483.	46-56